Rapid detection of hydrogen sulfide produced by pathogenic bacteria in focused growth media using SHS-MCC-GC-IMS

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Abstract

A new rapid method for the detection of hydrogen sulfide from pathogenic bacteria is reported. The developed method, static headspace – multi-capillary column - gas chromatography - ion mobility spectrometry (SHS-MCC-GC-IMS), has been applied to detect hydrogen sulfide evolution from 61 bacteria. The developed method has been compared against a standard triple sugar iron (TSI) agar approach, and a modified single sugar iron (SSI) agar approach. Hydrogen sulfide detection by SHS-MCC-GC-IMS using an initial inoculum of 1-1.5 x 10^5 CFU/mL can be achieved within 6 hours, after incubation at 37 °C, with a limit of detection of 1.6 ng/mL. Data for the standard agar method against the new instrumental approach, and the modified agar method against the new instrumental approach, are compared. The specificity for the new method compared against the standard method and the modified agar approach across all 61 strains was 85.2% and 88.5% respectively, and 86.7% and 91.3% across the 23 Salmonella strains tested.

Keywords: hydrogen sulfide; pathogenic bacteria; gas chromatography; ion mobility spectrometry.

Introduction

Hydrogen sulfide (H_2S) is a volatile compound with a characteristic rotten egg odour at low concentrations, and is commonly associated with bacterial contamination of food and water sources, particularly involving bacteria of the family *Enterobacteriaceae* [1]. It is one of the earliest volatile compounds identified as a product of microbial decomposition. One of the first documented studies of microbial

H_2S was published in 1875 [2]. The Doctoral Thesis [2] primarily focused on examining H_2S production from undefined microbes associated with chicken egg spoilage using lead acetate paper as the method of detection. Further work, during the same period, conducted by Orlowski (1895) [3] described H_2S production by *Typhoid bacillus*; according to current taxonomy this strain is likely to be a sub-species of *Salmonella enterica*. Further studies have examined H_2S production by *Salmonella* and other putrefactive organisms isolated from contaminated soil and faeces, and have been instrumental in the improvement of sanitation procedures for public drinking water [4-6]. Production of H_2S is particularly prevalent in members of the Gram-negative *Enterobacteriaceae* family, and is particularly associated with the *enterica* sub-species of the genus *Salmonella* [7]. However, H_2S has also been positively identified in *Citrobacter* spp*., Proteus* spp*., Edwardsiella* spp. [7], as well as in the non-*Enterobacteriaceae* Gram-negative bacterial genus *Shewanella,* which are often involved in marine carrion cycles [8]. The most infamous members of the *Enterobacteriaceae* family, *Escherichia coli* serotypes*,* are generally accepted as H_2S negative according to current testing methods [9]. However, many studies have shown positive H_2S production from *E. coli* strains isolated from various backgrounds. For example Lautrop *et al*. isolated 26 different H_2S positive *E. coli* strains from 25 different patients over a period of 9 months[10], a similar situation was also reported by Maker *et al.*[11] Furthermore, Magalhaes *et al.* [12] isolated positive H_2S producing *E. coli* strains from swine livestock. Clearly there is much contradiction throughout the literature regarding the H_2S production status of many bacteria, which when combined with a lack of recent studies into bacterial H_2S production, leads to a potentially misrepresented consensus.

Microbial sulfate reduction has been identified as one of the earliest complex biological pathways to develop, with isotopic sedimentary data indicating its emergence as early as 3.47 billion years ago [12]. Production of H_2S by microbes is a by-product of microbial anaerobic respiration, where sulfate is used in place of oxygen as a terminal electron acceptor [13]. Hydrogen sulfide production is highly variable throughout microorganisms at multiple taxonomic ranks, and there are multiple production pathways dependent on the type and concentration of the sulfur source present in their immediate environment. The main sources utilised by microbes for H_2S production are the sulfur containing amino acids cystine and

cysteine, and thiosulfate. Hydrogen sulphide production can also be achieved through utilisation of tetrathionate, sulfite, and sulfate, however this is less prevalent [14]. Cystine and cysteine are generally acquired through protein decomposition, whereas other sulfur containing compounds such as thiosulfate are generally found in anaerobic environments containing decaying organic matter, primarily soils and sea/river beds [15].

The pathway for cysteine utilisation for H_2S production has been somewhat explored, with cysteine desulfhydrase identified as the enzyme responsible [14], resulting in the formation of pyruvic acid, ammonia, and H_2S, which is then liberated as a gas [16]. However, there is also evidence that H_2S may be induced in response to excess cysteine as a protective mechanism against toxicity [17-19]. The enzyme responsible for thiosulfate utilisation has been identified as thiosulfate reductase, which reduces thiosulfate to sulfite and gaseous H_2S [20, 21].

Current tests employed for the detection of bacterial H_2S tend to rely on nutrient rich growth media supplemented with a sulfur source, usually including the addition of sodium thiosulfate, cystine, or cysteine hydrochloride, to induce significant production of H_2S [14]. These media are also combined with a visible colour change following incubation; usually facilitated via metallic salts, such as, ferric ammonium citrate or lead acetate, which forms a black precipitate with H_2S [7, 11]. One of the primary drawbacks of these methods is the subjective nature of the visual colour interpretation, which combined with relatively low sensitivity has meant that current methods have little application outside of differential taxonomic testing. This paper proposes a new method for the rapid and sensitive detection of H_2S using static headspace – multi capillary column - gas chromatography - ion mobility spectrometry (SHS-MCC-GC-IMS), with potential future application for detection of bacterial contamination in food and water sources, as well as detection of bacteria within various human clinical samples.

Experimental

Materials and Reagents

Meat extract, yeast extract, bacteriological peptone, lactose, tryptone soya agar, sodium sulphide (97%), hydrochloric acid, and Triple Sugar Iron agar were

purchased from Sigma-Aldrich (Dorset, UK). Sucrose, dextrose, sodium chloride, and sodium thiosulfate were purchased from Melford Laboratories Ltd. (Ipswich, UK).

Instrumentation

A static headspace-multi-capillary column-gas chromatography-ion mobility spectrometer (SHS-MCC-GC-IMS) manufactured by G.A.S.-Gesellschaft für Analytische Sensorsysteme mbH (Dortmund, Germany), was used.[22],[23] The instrument was fitted with an automatic sampler unit (CTC-PAL; CTC Analytics AG, Zwingen, Switzerland) and a heated gas-tight syringe. A multi-capillary column (MCC) (Multichrom, Novosibirsk, Russia) was used for the chromatographic separation. The MCC comprised a stainless steel tube, 20 cm × 3 mm ID, containing approximately 1000 parallel capillary tubes, 40 µm ID, coated with 0.2 µm film thickness of stationary phase (Carbowax 20M). Atmospheric pressure ionisation is generated by a Tritium (^{3}H) solid state bonded source (β-radiation, 100–300 MBq with a half-life of 12.5 years). The IMS has a drift tube length of 50 mm. Separation in the IMS drift tube is achieved by applying an electric field of 2 kV to the ionized volatiles in a pulsed mode using an electronic shutter opening time of 100 µs. The drift gas was N_2 (99.998%) with a drift pressure of 101 kPa (ambient pressure). Samples were run under the following operating conditions: incubation conditions (time, 3 min; and, temperature, 37 ^{o}C); MCC-IMS conditions (syringe temperature, 50°C; injection temperature, 80 ^{o}C; injection volume, 2.5 mL; column temperature, 40 ^{o}C; and, a column carrier gas flow programme rate, 5 mL/min with IMS conditions (temperature, 50 ^{o}C; and, drift gas flow rate, 500 mL/min). The total analysis time was 5 mins. All data was acquired in the negative ion mode and each spectrum is formed with the average of 12 scans. All data are processed using the LAV software (version 1.5.1, G.A.S). The experimental procedure has previously been reported for analysis of VOCs from bacteria [22-23].

Preparation of H_2S Standards

Initially, nitrogen gas was continuously bubbled through 0.01 M aqueous hydrochloric acid solution for 30 minutes to expel any dissolved oxygen within the acid, as oxygen interferes with the generation of gaseous H_2S. Then, H_2S standards were prepared by dissolving 0.01 g (accurately weighed) of sodium sulfide in 100 mL of the previously prepared 0.01M HCl solution, liberating H_2S gas to a stock

concentration of 0.1 mg/mL (100 µg/mL). From this stock solution, 1 mL was added to the previously prepared 0.01 M HCl solution to create a 1 µg/mL working solution. Using the working solution further dilutions were made in the concentration range 5 to 500 ng/mL and analysed via SHS-MCC-GC-IMS. Control samples of the 0.01 M HCl were run during the analyses of the standards, alongside TSI broth samples, to allow for blank subtraction. In addition, H_2S standards of 20, 40 and 60 ng/mL were prepared daily and ran on every test sampling day to compensate for any potential instrumental variance.

Microbiology

Bacteria used in this study were acquired from numerous sources, and are predominantly strains acquired from the National Collection of Type Cultures (NCTC) (Salisbury, UK) or other culture collections. Further wild and type culture strains were kindly provided by the Freeman Hospital, Newcastle UK, many of which were isolated from routine patient samples. Wild type *Escherichia coli* strains CPE 14/15/20 and ES 17/20 were named so due to their antimicrobial resistance profiles (CPE = carbapenemase producing *enterobacteriaceae,* ES = Extended spectrum β lactamase). All bacterial strains used are shown Table 1, along with their identification number where applicable. The majority of the bacterial strains used in this study were of the family *Enterobacteriaceae,* which were selected due to their high association with human pathogenicity, and also because of their role in food and water contamination [24]. Furthermore, many of the bacteria were selected due to their relevance regarding human pathogenicity and antimicrobial resistance, such as *Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa,* and *Enterobacter* spp*.,* organisms which are particularly relevant due to their status as ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa* and *Enterobacter species*) pathogens [25, 26].

TSI Broth Preparation

To prepare the TSI broth, 1 g of dextrose, 10 g of lactose, 10 g of sucrose, 3 g of meat extract, 3 g of yeast extract, 20 g of bacteriological peptone, 5 g of sodium chloride, and 0.3 g of sodium thiosulfate were dissolved in 1 L of distilled water and subsequently sterilized via an autoclave at 121˚C for 15 minutes. The recipe used for

this TSI broth mimics the TSI Agar recipe (Sigma-Aldrich, product code: 92499), with the omission of agar, phenol red, and ferric ammonium citrate.

Agar Slopes Preparation & Procedure

To prepare the TSI agar slopes, 64.6 g of the TSI agar powder (Oxoid, Basingstoke, UK) was added to 1 litre of deionised water (Milli-Q, Integral 3, 18 MΩ cm) and brought to boil using a hot plate with a built-in magnetic stirrer. SSI agar slopes were prepared using an identical composition to that of TSI agar slopes, with the omission of sucrose and lactose. For both agar types, 7 mL of the freshly boiled agar mixture was then aliquoted into 20 mL headspace vials (with lids loosely screwed on) and subsequently autoclaved at 121˚C for 15 minutes to achieve sterilization. The sterilized agar solutions were then removed and allowed to set at room temperature for approximately 30 minutes, whilst cooling the vials were positioned at an approximately 45˚ angle to allow for a sufficient slope to form. Once completely set, the agar slopes were either used immediately or refrigerated at 8˚C for future use.

To inoculate the agar slopes, colonies were picked from one-day old cultures on Tryptone soya agar plates and streaked thoroughly across the surface of the slopes, before stabbing through the agar to bottom of the tube (ensuring this step was only carried out once). The lids were screwed back on the vials, ensuring only a loose fit, and the vials were then placed in an incubator set to 37°C. The standard method for TSI agar slopes calls for the observations to be carried out only after 24 hours, however we also observed the slopes following 6 hours incubation, to allow a comparison with the new proposed method. Observations were also recorded on SSI agar slopes at 6, 24, and 96 hours incubation.

Bacterial Growth Conditions & Sample Preparation

Bacteria were cultured overnight on Tryptone Soya Agar at 37˚C one day prior to sample preparation. Following overnight incubation, fresh colonies were removed from the plates and inoculated into sterile TSI broth. The inoculated broth was adjusted to an absorbance of 0.132 at OD_{600nm} (equivalent to 0.5 McFarland units), giving an approximate cell suspension of 1-1.5 x 10^8 CFU / mL, 10 µL of the bacterial suspension was then added to a 20 mL clear headspace vial containing 9990 µL sterile TSI broth, giving an approximate final inoculation of 1-1.5 x 10^5 CFU / mL prior to incubation. The bacterial suspensions were then immediately sampled via

SHS-MCC-GC-IMS before being placed in an incubator set to 37˚C and were subsequently sampled every half hour for 8 hours, and sampled again after 24 hours incubation. Based on the result of the time study, a pre-incubation inoculum study was conducted where *Salmonella stanley* and *Salmonella typhimurium* suspensions of 1-1.5 x 10^2, 1-1.5 x 10^3,1-1.5 x 10^4, and 1-1.5 x 10^5 CFU / mL were created and analysed via SHS-MCC-GC-IMS after 6 hours incubation at 37˚C.

Results and Discussion

Initially the analytical performance of SHS-MCC-GC-IMS to detect H_2S in negative ion mode, was investigated. In negative ion mode fast electrons from the tritium β-radiation source react with the nitrogen carrier gas as follows:

$$N_2 + \beta \rightarrow N_2^+ + e^-$$

As air is present in the surrounding atmosphere further reactions take place with oxygen and water molecules to form the stable Reactant Ion Peak (RIP):

$$O_2 + e^- \rightarrow O_2^-$$

$$H_2O + O_2^- \rightarrow O_2^-.H_2O$$

$$H_2O + O_2^-.H_2O \rightarrow O_2^-(H_2O)_2$$

Subsequently the stable RIP ($O_2^-(H_2O)_2$) interacts with H_2S to form a cluster ion as follows:

$$O_2^-(H_2O)_2 + H_2S \rightarrow [H_2S.O_2^-(H_2O)_2]$$

The cluster ion ($[H_2S.O_2^-(H_2O)_2]$) subsequently stabilises to form the product ion (monomer) ($H_2S.O_2^-(H_2O)$):

$$[H_2S.O_2^-(H_2O)_2] \rightarrow H_2S.O_2^-(H_2O) + H_2O$$

Experimentally, H_2S had a monomer with a retention time of 5.35 ± 0.43 s and drift time of 5.75 ± 0.027 (n = 10) (Figure 1). The relative drift time ($t_{r.drift}$) for H_2S was calculated as follows:

$$t_{r.drift} = t_d / t_{d.RIP}$$

Where t_d is the measured drift time of H_2S (5.75 ms) and $t_{d.RIP}$ is the drift time of the reactant ion peak (RIP) in negative ion mode (0.187 ms). The normalised reduced ion mobility (Ko, $cm^2\ V^{-1}\ s^{-1}$) can then be calculated for H_2S. This was done by firstly calculating the normalised reduced ion mobility for the RIP ($Ko_{(RIP)}$):

$$Ko_{(RIP)} = [(L^2 / E \,.\, t_{D(RIP))} \,.\, (P / P_o) \,.\, (T_o / T)]$$

Where L is the length of the drift region (5 cm), E is the applied electrical field (2000 V), $t_{D(RIP)}$ is the drift time of the RIP (0.187 x 10^{-3} s), P is the pressure of the drift gas (hPa), P_o is the standard atmospheric pressure (1013.2 hPa), T is the temperature of the drift gas (323 K), and T_o is the standard temperature (273 K). The normalised reduced ion mobility for the RIP ($Ko_{(RIP)}$) was experimentally determined to be 56.50 $cm^2\ V^{-1}\ s^{-1}$ (n = 20). Subsequently, the normalised reduced ion mobility (Ko) for H_2S was then calculated:

$$K_{0(H2S)} = F_{IMS} / t_{D(H2S)}$$

Where F_{IMS} is the IMS factor ($cm^2\ V^{-1}$) which can be derived as follows: $F_{IMS} = K_{0(RIP)}$ x $t_{D(RIP)}$; where $Ko_{(RIP)}$ is 56.50 $cm^2\ V^{-1}\ s^{-1}$ and $t_{D(RIP)}$ (0.187 x 10^{-3} s). Finally, using the F_{IMS} value of 0.01056 $cm^{-1}\ V^{-1}$, the normalised reduced ion mobility for H_2S was calculated as 1.837 $cm^2\ V^{-1}\ s^{-1}$ (with a range of 1.828 – 1.845 $cm^2\ V^{-1}\ s^{-1}$).

The calibration data for H_2S was determined (Figure 2); a non-linear calibration graph for H_2S was determined over the concentration range 0 – 500 ng/mL. Quantification of bacterial samples was achieved using the equation $y = (ab + cx^d) / (b + x^d)$, where the coefficients: a = 51.57, b = 24.44, c = 11828, d = 1.2322 gave a regression coefficient, r, of 0.9981. The limit of detection (LOD) and limit of quantification (LOQ), based on 3 or 10 x standard deviation of the blank, respectively was determined. The limit of detection was experimentally determined to be 1.6 ng/mL while the limit of quantitation was experimentally determined as 5.5 ng/mL. In practice, the non-linear response for H_2S at the higher concentrations was addressed in this work as follows: any bacterial samples producing an H_2S concentration above 100 ng/mL were reported as >100 ng/mL.

To determine the optimal balance between incubation time and pre-incubation inoculum size, an initial study was conducted on four of the test bacteria. Specifically, *E. coli* NCTC 12241 and *E. coli K12* NCTC 8912 (both known to be H_2S negative according to standard testing); and, *S. Stanley,* and *S. typhimurium* (both known to be H_2S positive according to standard testing). An initial incubation time study, using SHS-MCC-GC-IMS, identified that bacteriologically produced H_2S could be detected in as little as 6 hours incubation with an initial inoculum of 1-1.5 x 10^5 CFU / mL (Figure 3). A further sample was also taken after 24 hours incubation and the determined H_2S concentrations were: *S. stanley* > 100 ng/mL; *S. typhimurium* >

100 ng/mL; *E. coli (NCTC 12241)* 12.1 ng/mL; and, *E. coli* (*K12 NCTC 8912*) 24.5 ng/mL. Based on these results and a 6 hour incubation period, a pre-incubation inoculum study was then conducted using S. stanley and S. typhimurium. The results (Figure 4) confirmed that the optimal initial inoculum for this incubation time period was 1-1.5 x 10^5 CFU / mL. Subsequent testing of the pathogenic bacteria was undertook, using SHS-MCC-GC-IMS, using a 6 hour incubation time with an initial inoculum of 1-1.5 x 10^5 CFU/mL.

Triple Sugar Iron (TSI) agar slopes were developed primarily for the differentiation of *Enterobacteriaceae* strains, and therefore are not used solely for detection of H_2S production. Other differential components included in TSI agar are glucose, sucrose, and lactose to assess microbial carbohydrate fermentation, as well as a pH indicator. The results of these aspects of the TSI agar test were deemed beyond the scope of this study, and therefore only the result of H_2S production testing has been recorded. A disadvantage of TSI agar, in terms of monitoring H_2S production, is the potential for products of carbohydrate fermentation interfering with the precipitate formation reaction between H_2S and ferric ammonium citrate, thereby producing false negative results [27]. Due to this potential for erroneous results with this agar, it was decided to modify the composition of TSI agar to remove sucrose and lactose, creating single sugar iron (SSI) agar to be tested alongside the standard method, in comparison to our proposed analytical technique. Table 1 shows all the bacteria tested for H_2S production by traditional agar, the modified agar, and the proposed method. A total of 61 bacterial isolates were tested, all in duplicate. Of the 61 isolates tested, all were potential human pathogens, and 56 of which were members of the *Enterobacteriaceae* family. The results from the agar slopes were recorded following the recommended 24 hour incubation period, as well as after 6 hours incubation in order to compare against the incubation period utilised in the SHS-MCC-GC-IMS method. The SSI agar slope results were also recorded after 96 hours incubation.

When compared with the standard method for H_2S using TSI agar slopes, and a 24 hour incubation, the new proposed 6 hour SHS-MCC-GC-IMS method was in agreement (with either positive or negative identification), on the basis of H_2S production, in 85.2% (52/61) of the Gram-negative bacteria tested, and 14.8% (9/61) gave a different response for H_2S than expected. Similarly, if the comparison with the

TSI agar slopes, based on a 6 hour incubation, is used to compare to the 6 hour SHS-MCC-GC-IMS then 86.9% (53/61) of the Gram-negative bacteria tested are in agreement, and 13.1% (8/61) gave a different response for H_2S than expected. When the analytical method was compared against SSI agar slopes, agreement on H_2S production was recorded in 88.5% (54/61) of the 61 bacteria tested, with differences coming from *Salmonella Indiana* and *Enterobacter cloacae*, which are known to ferment lactose [28] and sucrose [29], respectively.

Of the 23 *Salmonella* strains tested on the standard 24 h TSI agar slopes, 20 were found to be H_2S positive, to varying degrees (identified as significant-to-complete coverage of the agar) (Table1). Interestingly, the same results were also observed following just 6 hours incubation albeit of a lower threshold determination (i.e. a slight black precipitate). In the case of *S. stanley* however, only trace H_2S was detected after 6 hour incubation on the agar slopes. Furthermore, when the same 23 *Salmonella* isolates were tested via SHS-MCC-GC-IMS, 22/23 tested positive for H_2S production, with only *Salmonella gallinarum* producing definitive H_2S negative results on all testing methods. The Salmonella strains *othmarschen, hadar, saint-paul,* and *derby* all produced H_2S positive results on slopes whilst producing less than 1.6 ng/ml (<LOD) according to our analytical method; a potential explanation for this could be that the 6 hours incubation utilised was not sufficient for these strains to produce a significant quantity of H_2S. Interestingly, *Salmonella senftenburg* was consistently H_2S negative on slopes, but produced a small signal for H_2S according to our method (<LOD); potentially highlighting an issue with indicator sensitivity in the TSI medium. A further difference was noted for *S. indiana* which produced a positive response for H_2S (9.8 ng/mL), following 6 hours incubation, with detection by SHS-MCC-GC-IMS, contrasting with the H_2S negative response observed on TSI slopes. This is due to the ability of *S. indiana* to ferment lactose [28], the product of which has been shown to mask precipitate formation [30], therefore producing a false negative for H_2S production on TSI agar slopes. This is compounded in the H_2S production observed in *S. Indiana* on SSI agar slopes. Furthermore, the omission of ferric ammonium citrate from the TSI broth thereby allows any H_2S produced to be released into the gaseous headspace and detected via SHS-MCC-GC-IMS.

Results obtained from the *Citrobacter freundii* and the *Proteus* isolates tested were all encouraging, showing that analysis via SHS-MCC-GC-IMS was able to consistently detect H_2S production after just 6 hours incubation at 37°C with a low initial inoculum of 1-1.5 x 10^5 CFU / mL. Interestingly, it was observed that despite the far higher inoculum, both *Citrobacter freundii* isolates performed poorly on the TSI agar slope observations after 6 hours incubation, with *C. freundii* NCIMB 8645 only producing trace H_2S on both samples, and *C. freundii* NCTC 9750 only producing trace H_2S in one sample. A possible explanation could be the ability of these strains to ferment sucrose and/or lactose, a notion which is supported by these strains increased H_2S production activity on SSI agar slopes.

All isolates of the following genera tested negative on both TSI agar slopes (at both incubation times) and using SHS-MCC-GC-IMS detection; *Escherichia*, *Klebsiella, Serratia, Yersinia, Hafnia, Stenotrophomonas, Acinetobacter,* and *Burkholderia.* Of the four *Shigella* species tested, only *Shigella sonnei* tested positive for H_2S via SHS-MCC-GC-IMS, producing an average concentration of 7.6 ng/mL. This could therefore have potential as a method for the differentiation of *Shigella sonnei* from other *Shigella* species.

In both *Pseudomonas aeruginosa* isolates, no H_2S was detected after 6 hours incubation on slopes or using SHS-MCC-GC-IMS. Trace H_2S was detected for both strains following 24 hours incubation on both TSI and SSI agar slopes, indicating that while they possess potential for H_2S production; a significant incubation period and inoculum are required, precluding its use as an identifying biochemical characteristic.

Conflicting results were also observed by SHS-MCC-GC-IMS analysis of *Cronobacter sakazakii* ATCC 29544*, Providencia rettgeri* NCTC 7475, and *Providencia stuartii* NCTC 10318, which all tested H_2S positive. *Cronobacter sakazakii* is known to be able to ferment both lactose [31] and sucrose [32], however *C. sakazakii* was not observed to produce H_2S on SSI agar at either 6 or 24 hours incubation, but did produce trace H_2S after 96 hours incubation. No difference was noted between TSI and SSI agar slopes for either *Providencia* strain, concluding that sugar fermentation activity had no effect on H_2S detection. These findings therefore suggests that whilst these strains possess potential for H_2S production, a significant

incubation period and inoculum are required, precluding its use as an identifying biochemical characteristic.

A surprising result is the minimal evolution of H_2S (<LOD) according to SHS-MCC-GC-IMS detection by *Edwardsiella tarda* NCTC 11934. This contrasted with the strongly positive H_2S production observed on TSI agar slope following 24 hours incubation. The low H_2S responses using both approaches at 6 hour incubation are probably linked to the somewhat fastidious nature of *E. tarda*; a predominantly marine pathogen suited to lower incubation temperatures. *E. tarda* has however been documented to have serious human pathogenic capability, and therefore should not be discounted [33].

It is worth noting that the pre-incubation inoculum used in agar slopes is significantly higher than used in the new proposed SHS-MCC-GC-IMS method (1-1.5 x 10^5 CFU/mL). Where the standard TSI method may use 1 colony per slope, our proposed method requires roughly one colony to be diluted and homogenized in approximately 7 mL of broth, of which only 10 µL is diluted into 9990 µL of broth. Furthermore, the low initial CFU/mL required to analyse H_2S by SHS-MCC-GC-IMS is more representative of an *in vivo Salmonella* infection [34], which when combined with the rapid run time of 6 hours could potentially mean this test could be performed in a clinical laboratory within the confines of a normal 'working day'.

Conclusion

A new method to detect bacteria generated H_2S is proposed based on SHS-MCC-GC-IMS. Analysing the headspace above a bacterial suspension with an initial inoculum of 1-1.5 x 10^5 CFU / mL after 6 hours of incubation at 37°C is a relatively rapid and extremely sensitive method for the detection of bacteriologically produced H_2S, allowing for detection as low as 1.6 ng/mL. A major advantage of this new, rapid method over the current agar based colorimetric methods is the potential for a clinical sample to be collected, cultured, and analysed within a working day. This method is particularly useful for analysing bacteria which do not have strict growth requirements, such as *Salmonella* spp*., Citrobacter* spp*.,* and *Proteus* spp*.* However, the developed approach could be extended by incorporating a more flexible

(increased) incubation period to suit the target organism. This new rapid method could potentially be applied for the analysis of various clinical or food samples.

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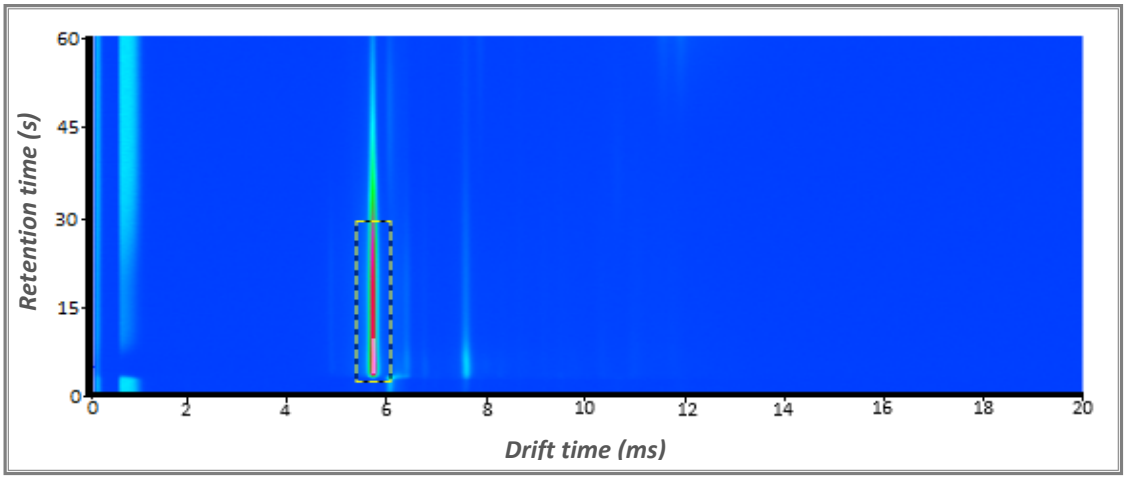
Figure 1. Data Visualisation for H_2S determined by MCC-GC-IMS (a) topographical view showing monomer and (b) 2-dimensional view

(a)



(b)

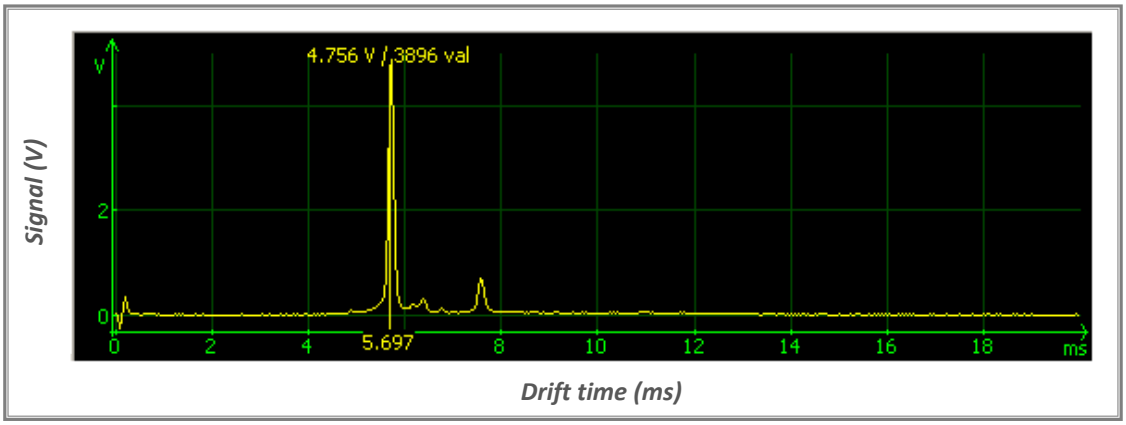


Figure 2. Calibration Graph for H_2S by SHS-MCC-GC-IMS

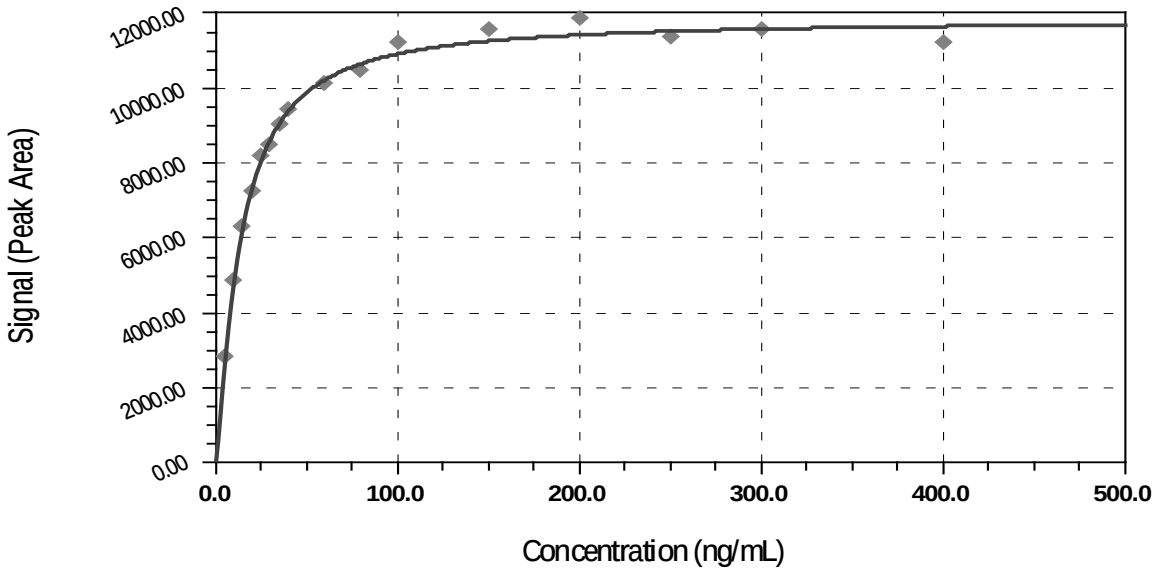
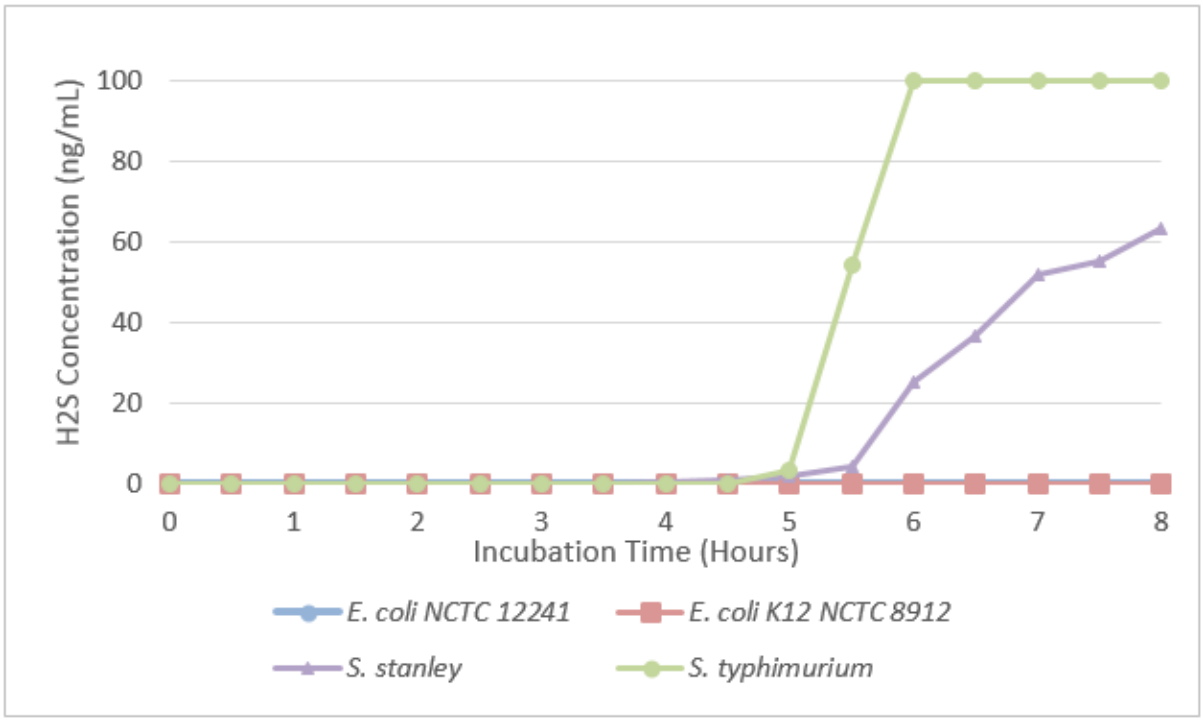


Figure 3. Investigation of incubation time for known positive and negative H_2S-producing bacteria by SHS-MCC-GC-IMS.

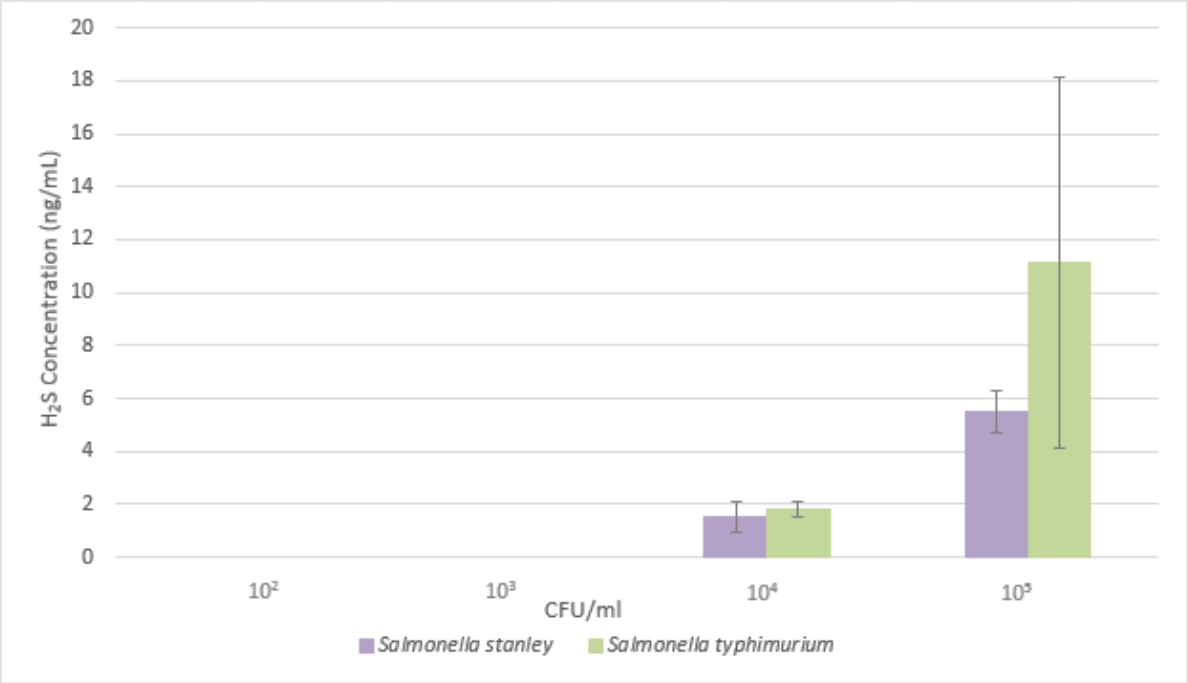


Notes:

Monitoring of H_2S production by *E. coli* and *Salmonella* strains over an 8 hour incubation period at 37˚C. Headspace sampling was done every 30 minutes up to an incubation time of 8 hours (a further sample was also taken after 24 hours incubation).

Values displayed are the average of two separate bacterial suspensions per strain, set up identically, and are presented in ng/mL.

Figure 4. Pre-incubation inoculum optimization



Note:

Values displayed are the average of two separate bacterial suspensions per strain, set up identically, and are presented in ng/mL (± sd).

Table 1. Comparison of 61 Gram-negative bacteria tested for H_2S production using the traditional triple sugar iron (TSI) agar slopes, a modified single sugar iron (SSI) agar slopes and SHS-MCC-GC-IMS

Taxonomic hierarchy				TSI Agar Slopes		SSI Agar Slopes			SHS-MCC-GC-IMS	SHS-MCC-GC-IMS data compared to Standard TSI Method at 24 hour incubation@	SHS-MCC-GC-IMS data compared to SSI Agar Method at 24 hour incubation@
Family	**Genus**	**Species**	**Serovar / ID number**	**6 hour incubation**	**24 hour incubation**	**6 hour incubation**	**24 hour incubation**	**96 hour incubation**	**6 hour incubation (ng/mL)**		
Enterobacteriaceae	*Salmonella*	*enterica*	*stanley (Wild)*	**T** (T, T)	**+++** (++++, +++)	**+** (+, +)	**++++** (++++, ++++)	**++++** (++++, ++++)	**10.5** (10.6, 10.7)	√	√
			london (Wild)	**+** (+, +)	**+++** (++++, +++)	**+** (+, +)	**++++** (++++, ++++)	**++++** (++++, ++++)	**8.7** (8.0, 9.3)	√	√
			gallinarum (Wild)	**–** **(-, -)**	– (-, -)	**–** **(-, -)**	**–** **(-, -)**	**–** **(-, -)**	**0.0** (0.0, 0.0)	√	√
			othmarschen (Wild)	**+** (+, +)	**+++** (++++, +++)	**+** (+, +)	**++++** (++++, ++++)	**++++** (++++, ++++)	**< LOD**	√	√
			oranienburg (Wild)	**+** (+, +)	**+++** (++++, +++)	**+** (+, +)	**++++** (++++, ++++)	**++++** (++++, ++++)	**55.7** (63.1, 48.4)	√	√
			typhimurium (Wild)	**+** (+, +)	**++++** (++++, ++++)	**+** (+, +)	**++++** (++++, ++++)	**++++** (++++, ++++)	**26.0** (29.0, 22.9)	√	√
			javiana (Wild)	**+** (+, +)	**+++** (++++, +++)	**+** (+, +)	**++++** (++++, ++++)	**++++** (++++, ++++)	**14.3** (13.6, 15.1)	√	√
			hadar (Wild)	**+** (+, +)	**+++** (++++, +++)	**+** (+, +)	**++++** (++++, ++++)	**++++** (++++, ++++)	**< LOD**	√	√
			indiana (Wild)	**–** **(-, -)**	– (-, -)	**+** (+, +)	**++++** (++++, ++++)	**++++** (++++, ++++)	**9.8** (12.6, 7.0)	X	√
			zanzibar (Wild)	**+** (+, +)	**+++** (++++, +++)	**+** (+, +)	**++++** (++++, ++++)	**++++** (++++, ++++)	**25.5** (35.2, 15.9)	√	√
			braenderup (Wild)	**+** (+, +)	**++++** (++++, ++++)	**+** (+, +)	**++++** (++++, ++++)	**++++** (++++, ++++)	**36.8** (41.6, 32.1)	√	√
			vilvoorde (Wild)	**+** (+, +)	**+++** (++++, +++)	**+** (+, +)	**++++** (++++, ++++)	**++++** (++++, ++++)	**26.1** (27.7, 24.4)	√	√
			agona (Wild)	**+** (+, +)	**++++** (++++, ++++)	**+** (+, +)	**++++** (++++, ++++)	**++++** (++++, ++++)	**45.8** (48.5, 43.2)	√	√
			Muenchen (Wild)	**+** (+, +)	**+++** (+++, +++)	**+** (+, +)	**++++** (++++, ++++)	**++++** (++++, ++++)	**10.1** (10.8, 9.4)	√	√
			saint-paul (Wild)	**+** (+, +)	**+++** (+++, +++)	**+** (+, +)	**++++** (++++, ++++)	**++++** (++++, ++++)	**< LOD**	√	√
			abony (Wild)	**+** (+, +)	**+++** (++++, +++)	**+** (+, +)	**++++** (++++, ++++)	**++++** (++++, ++++)	**48.9** (48.5, 49.3)	√	√
			bareilly (Wild)	**+** (+, +)	**++++** (++++, ++++)	**+** (+, +)	**++++** (++++, ++++)	**++++** (++++, ++++)	**44.0** (32.2, 55.8)	√	√

			meleagridis (Wild)	**+** (+, +)	**++++** (++++, ++++)	**+** (+, +)	**++++** (++++, ++++)	**++++** (++++, ++++)	**44.4** (42.2, 46.5)	√	√
			derby (Wild)	**+** (+, +)	**+++** (+++, +++)	**+** (+, +)	**++++** (++++, ++++)	**++++** (++++, ++++)	**< LOD**	√	√
			augustenborg (Wild)	**+** (+, +)	**+++** (+++, +++)	**+** (+, +)	**++++** (++++, ++++)	**++++** (++++, ++++)	**1.9** (1.8, 1.9)	√	√
			montevideo (Wild)	**+** (+, +)	**+++** (++++, +++)	**+** (+, +)	**++++** (++++, ++++)	**++++** (++++, ++++)	**20.9** (18.2, 23.6)	√	√
			senftenburg (NCTC 9959)	**–** **(-, -)**	– (-, -)	**–** **(-, -)**	**–** **(-, -)**	**–** **(-, -)**	**< LOD**	X	X
			Enteritidis (NCTC 6676)	**+** (+, +)	**+++** (++++, +++)	**+** (+, +)	**++++** (++++, ++++)	**++++** (++++, ++++)	**30.2** (26.1, 34.3)	√	√
	Citrobacter	*freundii*	*NCIMB 8645*	**T** (T, T)	**+++** (++++, +++)	**+** (+, +)	**++++** (++++, ++++)	**++++** (++++, ++++)	**> 100** (>100, > 100)	√	√
			NCTC 9750	**–** (-, T)	**++** (++, +++)	**+** (+, +)	**++++** (++++, ++++)	**++++** (++++, ++++)	**46.1** (44.7, 47.5)	√	√
	Escherichia	*coli*	*NCTC 12241*	**–** **(-, -)**	– (-, -)	**–** **(-, -)**	**–** **(-, -)**	**–** **(-, -)**	**0.0** (0.0, 0.0)	√	√
			NCTC 8912	**–** **(-, -)**	– (-, -)	**–** **(-, -)**	**–** **(-, -)**	**–** **(-, -)**	**0.0** (0.0, 0.0)	√	√
			O157 (NCTC 12079)	**–** **(-, -)**	– (-, -)	**–** **(-, -)**	**–** **(-, -)**	**–** **(-, -)**	**0.0** (0.0, 0.0)	√	√
			CPE 14 (Wild)	**–** **(-, -)**	– (-, -)	**–** **(-, -)**	**–** **(-, -)**	**–** **(-, -)**	**0.0** (0.0, 0.0)	√	√
			CPE 15 (Wild)	**–** **(-, -)**	– (-, -)	**–** **(-, -)**	**–** **(-, -)**	**–** **(-, -)**	**0.0** (0.0, 0.0)	√	√
			CPE 20 (Wild)	**–** **(-, -)**	– (-, -)	**–** **(-, -)**	**–** **(-, -)**	**–** **(-, -)**	**0.0** (0.0, 0.0)	√	√
			ES 17 (Wild)	**–** **(-, -)**	– (-, -)	**–** **(-, -)**	**–** **(-, -)**	**–** **(-, -)**	**0.0** (0.0, 0.0)	√	√
			ES 20 (Wild)	**–** **(-, -)**	– (-, -)	**–** **(-, -)**	**–** **(-, -)**	**–** **(-, -)**	**0.0** (0.0, 0.0)	√	√
	Shigella	*Dysenteriae (type 3)*	*NCTC 9730*	**–** **(-, -)**	– (-, -)	**–** **(-, -)**	**–** **(-, -)**	**–** **(-, -)**	**0.0** (0.0, 0.0)	√	√
		sonnei	*NCTC 9774*	**–** **(-, -)**	– (-, -)	**–** **(-, -)**	**–** **(-, -)**	**–** **(-, -)**	**7.6** (7.5, 7.7)	X	X
		boydii	*NCTC 9327*	**–** **(-, -)**	– (-, -)	**–** **(-, -)**	**–** **(-, -)**	**–** **(-, -)**	**0.0** (0.0, 0.0)	√	√
		flexneri	*NCTC 9780*	**–** **(-, -)**	– (-, -)	**–** **(-, -)**	**–** **(-, -)**	**–** **(-, -)**	**0.0** (0.0, 0.0)	√	√
	Enterobacter	*cloacae*	*NCTC 11936*	**–** **(-, -)**	– (-, -)	**–** **(-, -)**	**+** (+, +)	**+** (+, +)	**1.9** (2.6, 1.2)	X	√
		aerogenes	*NCTC 9777*	**–** **(-, -)**	– (-, -)	**–** **(-, -)**	**–** **(-, -)**	**–** **(-, -)**	**0.0** (0.0, 0.0)	√	√

	Klebsiella	*pneumoniae*	*Wild*	**–** **(-, -)**	– (-, -)	**–** **(-, -)**	**–** **(-, -)**	**–** **(-, -)**	**0.0** (0.0, 0.0)	√	√
			NCTC 243	**–** **(-, -)**	– (-, -)	**–** **(-, -)**	**–** **(-, -)**	**–** **(-, -)**	**0.0** (0.0, 0.0)	√	√
			NCTC 418	**–** **(-, -)**	– (-, -)	**–** **(-, -)**	**–** **(-, -)**	**–** **(-, -)**	**0.0** (0.0, 0.0)	√	√
			NCTC 9633	**–** **(-, -)**	– (-, -)	**–** **(-, -)**	**–** **(-, -)**	**–** **(-, -)**	**0.0** (0.0, 0.0)	√	√
	Cronobacter	*sakazakii*	*ATCC 29544*	**–** **(-, -)**	– (-, -)	**–** **(-, -)**	**–** **(-, -)**	**T** (T, T)	**11.1** (10.4, 11.7)	X	X
	Serratia	*marcescens*	*NCTC 10211*	**–** **(-, -)**	– (-, -)	**–** **(-, -)**	**–** **(-, -)**	**–** **(-, -)**	**0.0** (0.0, 0.0)	√	√
		odorifera	*NCTC 11214*	**–** **(-, -)**	– (-, -)	**–** **(-, -)**	**–** **(-, -)**	**–** **(-, -)**	**0.0** (0.0, 0.0)	√	√
		liquefaciens	*NCTC 11361*	**–** **(-, -)**	– (-, -)	**–** **(-, -)**	**–** **(-, -)**	**–** **(-, -)**	**0.0** (0.0, 0.0)	√	√
	Yersinia	*enterocolitica*	*NCTC 11176*	**–** **(-, -)**	– (-, -)	**–** **(-, -)**	**–** **(-, -)**	**–** **(-, -)**	**0.0** (0.0, 0.0))	√	√
		pseudotuberculosis	*NCTC 10275*	**–** **(-, -)**	– (-, -)	**–** **(-, -)**	**–** **(-, -)**	**–** **(-, -)**	**0.0** (0.0, 0.0)	√	√
	Hafnia	*alvei*	*NCTC 8105*	**–** **(-, -)**	– (-, -)	**–** **(-, -)**	**–** **(-, -)**	**–** **(-, -)**	**0.0** (0.0, 0.0)	√	√
	Edwardsiella	*tarda*	*NCTC 11934*	**+** (+, +)	**++++** (++++, ++++)	**+** (+, +)	**++++** (++++, ++++)	**++++** (++++, ++++)	**< LOD**	√	√
	Proteus	*vulgaris*	*NCTC 4175*	**+** (+, +)	**+++** (++++, +++)	**+** (+, +)	**++++** (++++, ++++)	**++++** (++++, ++++)	**2.0** (2.1, 1.9)	√	√
		mirabilis	*NCTC 10975*	**+** (+, +)	**+++** (++++, +++)	**+** (+, +)	**++++** (++++, ++++)	**++++** (++++, ++++)	**74.1** (89.3, 58.8)	√	√
			NCTC 11938	**+** (+, +)	**+++** (++++, +++)	**+** (+, +)	**++++** (++++, ++++)	**++++** (++++, ++++)	**22.8** (23.9, 21.7)	√	√
	Providencia	*stuartii*	*NCTC 10318*	**–** **(-, -)**	– (-, -)	**–** **(-, -)**	**–** **(-, -)**	**–** **(-, -)**	**6.7** (6.5, 7.0)	X	X
		rettgeri	*NCTC 7475*	**–** **(-, -)**	– (-, -)	**–** **(-, -)**	**–** **(-, -)**	**–** **(-, -)**	**4.4** (4.3, 4.2)	X	X
Pseudomonadaceae	*Pseudomonas*	*aeruginosa*	*NCTC 8295*	**–** **(-, -)**	**T** (T, T)	**–** **(-, -)**	**+** (+, +)	**+** (+, +)	**0.0** (0.0, 0.0)	X	X
			DSMZ 19880	**–** **(-, -)**	**T** (T, T)	**–** **(-, -)**	**+** (+, +)	**+** (+, +)	**0.0** (0.0, 0.0)	X	X
Xanthomonadaceae	*Stenotrophomonas*	*maltophilia*	*NCTC 10257*	**–** **(-, -)**	– (-, -)	**–** **(-, -)**	**–** **(-, -)**	**–** **(-, -)**	**0.0** (0.0, 0.0)	√	√
Moraxellaceae	*Acinetobacter*	*baumanii*	*ATCC 19606*	**–** **(-, -)**	– (-, -)	**–** **(-, -)**	**–** **(-, -)**	**–** **(-, -)**	**0.0** (0.0, 0.0)	√	√
Burkholderiaceae	*Burkholderia*	*cepacia*	*ATCC 25416*	**–** **(-, -)**	– (-, -)	**–** **(-, -)**	**–** **(-, -)**	**–** **(-, -)**	**0.0** (0.0, 0.0)	√	√

Notes:

TSI and SSI agar slope results were recorded according to the following criteria: - = No black precipitate formation; T = Trace black precipitate formation; + = Slight black precipitate formation; ++ = Significant black precipitate, covering less than 50% of the agar; +++ = Significant black precipitate, covering more than 50% of the agar but not complete coverage; and, ++++ = Complete coverage of the agar.

SHS-MCC-GC-IMS test results were recorded as the concentration of H_2S detected in ng/mL, all isolates were tested in duplicate with both values displayed in brackets below the average value in bold. H_2S concentrations below 1.6 ng/mL were classed as below the limit of detection and labelled as < LOD, and those which exceeded the quantification threshold are labelled as >100 ng/mL.

@ '√' results concur between TSI/SSI agar (24 hours) and SHS-MCC-GC-IMS (6 hours) and include results for which <LOD has been recorded; while 'X' results are considered to contradict each other between TSI and/or SSI agar (24 hours) and SHS-MCC-GC-IMS (6 hours).